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Isolation of a fungus from shell lesions of New Zealand abalone, *Haliotis iris* Martyn and *H. australis* Gmelin

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Abstract

Shell irregularities characterized by deposition of conchiolin on the inner shell surface of abalone, *Haliotis* spp., have been reported in populations along the South Island of New Zealand for over 50 years. A previously reported, but unidentified, fungus associated with these shell lesions is isolated for the first time from *Haliotis iris*, the black footed paua, and *H. australis*, the yellow footed paua, both obtained from Jacks Bay, South East Otago. Fungal hyphae measured 1.20 - 2.38 µm ($\bar{x}$ = 1.83 µm) in diameter. Occasionally, wider hyphae that measured 2.38 - 3.60 µm ($\bar{x}$ = 2.60 µm) in diameter with an apical swelling that measured 4.80 - 7.20 x 7.20 - 15.00 µm ($\bar{x}$ = 6.20 x 11.80 µm) were observed within lesions. Visible growth of aerial hyphae was observed on plates within 12 d at 16 °C on several media. Three morphologically indistinguishable isolates were obtained: two originated from *H. iris* shells and one from an *H. australis* shell. We examined 8 fungal and/or bacterial media and determined that the fungus grew best on a dilute medium (yeast extract, glucose, shell extract (+/-peptone agar) at 15 °C. Fungal growth was observed between 7 – 20 °C but not at room temperature (24 °C). Sporulation has not been induced, therefore the fungus is provisionally identified as a member of the Class Deuteromycotina.

No significant relationship between animal length and incidence of shell mycosis was observed. Abalone condition was not influenced by the shell mycosis in either species. The number of circulating haemocytes was significantly elevated in infected *H. australis* (1.19×10^7 cells ml^{-1}) relative to uninfected individuals (6.72×10^6 cells ml^{-1}) but not between infected and uninfected *H. iris*. No significant relationships were observed between the degree of fungal infection and protein levels of whole haemolymph, serum or sex (immature, male or female; $p<0.050$; N=34). We are currently attempting to determine the taxonomic placement of the fungus and further characterize its growth physiology and pathogenicity.

Introduction

Shell deformities characterized by the deposition of conchiolin on the dorsal region of the inner surface of the shell of abalone, *Haliotis* spp., have been reported in populations along the South Island of New Zealand for over 50 years (Sinclair 1963; Sainsbury 1977; Grindley unpublished data). Grindley (unpublished data) observed the presence of an unidentified fungus within shell lesions of affected abalone. Prior to our characterization, only two fungal species, *Haliphthoros milfordensis* Vishniac, 1958 and *Atkinsiella awabi* Kitancharoen, Nakamura, Wada and Hatai, 1994, have been described and/or isolated from abalone. Both of these fungal pathogens were associated with lesions in the soft tissues of abalone; *H. sieboldii* Reeve, 1846, from Japan. *Haliphthoros milfordensis* caused tubercle-like or flat lesions on the mantle, epipodium and dorsal surface of the

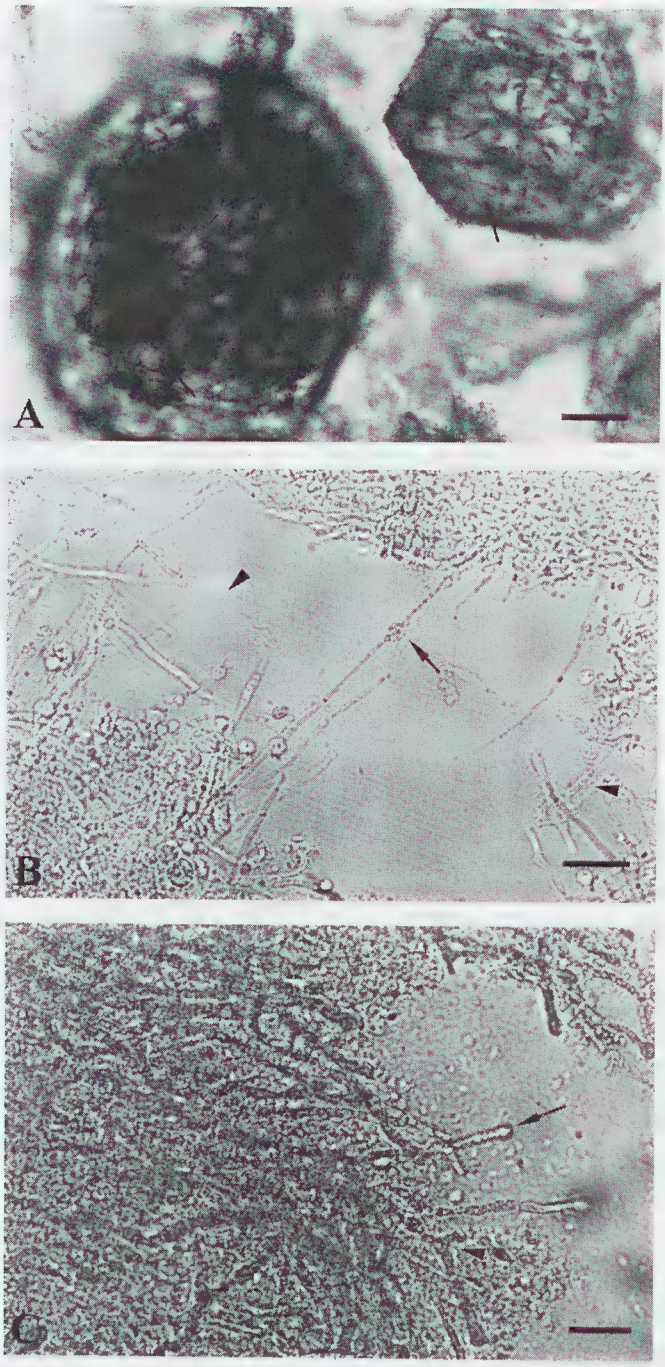

Figure 1 Lesion squashes. A. Polygonal conchiolin bodies from an advanced lesion penetrated with numerous fungal hyphae (arrows). Phase contrast. Bar = 22 µm. B. Wet mount of hyphae in a newly formed lesion. Note the septate nature of the fungus (arrow heads) and intercalary chlamydospore (arrow). Phase contrast. Bar = 16 µm. C. Wet mount of wider hyphae within a newly formed lesion . Arrow head denotes septa and a mycelial branch. Terminal swellings (arrow head) were frequently observed. Phase contrast. Bar = 15 µm.

foot (Hatai 1982). Clinical presentations of *A. awabi* resembled those of *H. milfordensis* and were described as raised tubercle-like lesions on the mantle and melanized lesions on the foot (Kitancharoen *et al.* 1994). Although marine fungi have been reported as invaders of calcareous materials such as barnacle tests and molluscan shells (e.g. *Pharcidia balani* (Winter) Bauch, 1936), no marine fungi have been identified or isolated from abalone shells (Kohlmeyer & Kohlmeyer 1979). We report the first isolation of a pathogenic fungus from shells of abalone from New Zealand. We isolated a fungus and characterized its growth characteristics. This study also assessed whether the shell mycosis affected the health of abalone as measured by a condition index and several hemolymph parameters.

Materials and methods

Abalone

Haliotis iris, the black footed paua, and *H. australis*, the yellow footed paua, were collected from intertidal and subtidal locations in Jack's Bay, Otago, New Zealand, on 3 November, 1995. Abalone were transported to the Portobello Marine Laboratory, University of Otago, and placed in two aquaria that received sand-filtered, full strength seawater at 13 °C. Abalone were fed *Macrocystis pyrifera* Bory, 1826, *ad libitum*. After tagging individuals, total weight (TW), shell weight (SW) and length (L) were determined prior to examination. A condition index (CI) was calculated for each animal: CI = {(TW-SW)/TW}. An aliquot of hemolymph was aseptically aspirated from the pallial sinus. The density of circulating haemocytes was quantified using a hemocytometer, and protein levels of whole haemolymph were determined according to the methods of Lowry (1951). In addition, an aliquot of whole hemolymph was centrifuged at 325 x g for 5 min, and the serum was analyzed for total protein as above. The shell of each abalone was removed and examined for lesions. Presence of lesions was quantified according to the following 0 to 3+ scale: 0) no lesion present; 1) lesion measured < 1 cm^2; 2) lesion measured 1–3 cm^2; 3) lesion measured > 3 cm^2. Tissues and shell adjacent to lesions were examined visually for penetration of fungal hyphae, and selected tissues were processed for routine paraffin histology.

Fungal Isolation

Shells with lesions characteristic of the abalone shell mycosis were surface sterilized with betadine and 70 % ethanol, followed by a rinse with 0.2 µm-filtered seawater. Small pieces of lesions were excised, placed on a glass slide and viewed by phase contrast microscopy (Figure 2). Soft lesions with visible hyphae were inoculated onto the following 4 media: 1) modified Vishniac medium (Shields 1990) supplemented with 100 ml l^{-1} of 10% (w/v) autoclaved (15 psi, 1 h) shell extract (MMV); 2) Glucose-yeast agar (Alderman & Polglase 1986) supplemented with 500 mg ml^{-1} Penicillin G, 500 mg ml^{-1} Streptomycin and 40 ml l^{-1} of 10% (w/v) shell extract (IM); 3) Sabauroud Dextrose Agar (Difco Laboratories, Detroit, Mich.) made with 50% seawater; 4) Marine agar (Difco Laboratories, Detroit, Mich.) supplemented with 40 ml l^{-1} of 10% (w/v) shell extract (MA). Plates were incubated at 15–16 °C under aerobic and microaerophilic conditions (using a candle jar) for up to 2 months.

Fungal Culture Conditions

To assess the best growth medium, colonies were subcultured in triplicate onto the following six media and incubated at 15 °C: MMV, IM, Brain Heart Infusion Agar (Difco Laboratories, Detroit, Mich.) with and without 2% added salt (BHI (+/-2% NaCl), Corn Meal Agar +2% NaCl (CMA, Difco Laboratories, Detroit, Mich.), and Potato Dextrose Agar +2% NaCl (PDA, Difco Laboratories, Detroit, Mich.). Colonies were also subcultured onto MMV, IM, BHI, and BHI+2% NaCl and incubated at 7, 15, 20 and 24 °C. Colony radius was measured to the nearest 0.1 mm every 1–3 wk to quantify relative growth rates at each of the four experimental temperatures. We attempted to induce

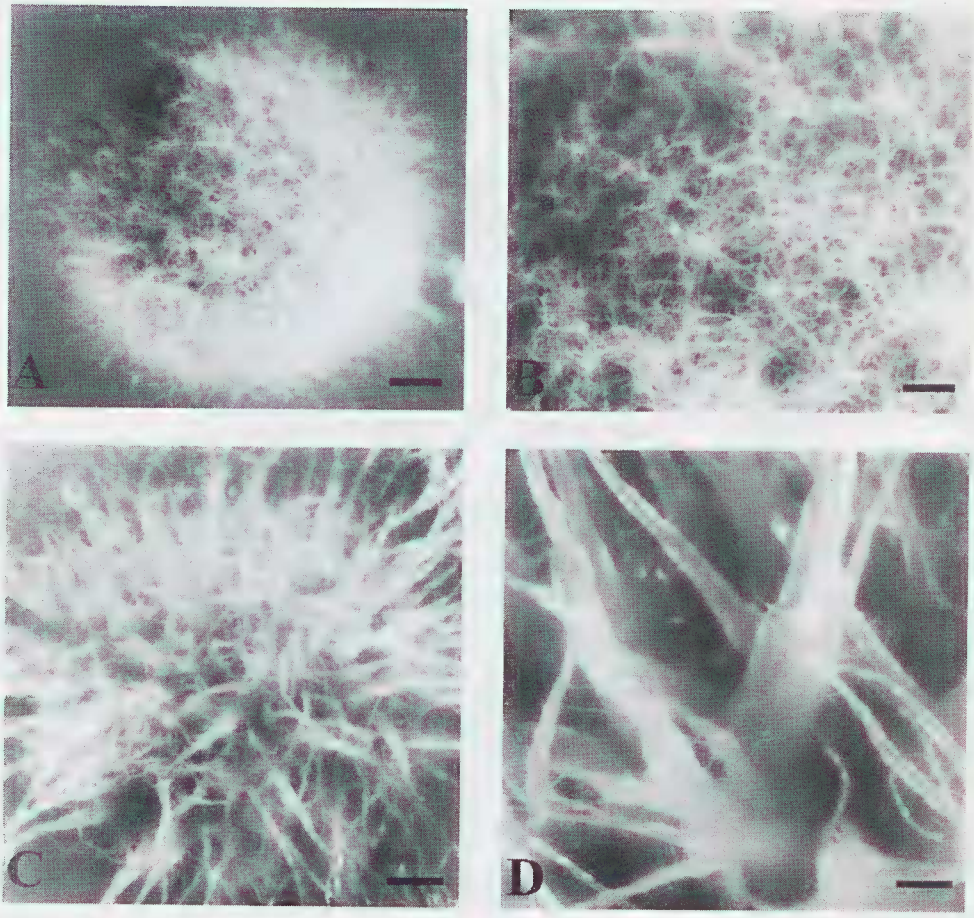

Figure 2 Two colony morphologies isolated in pure culture from abalone shell lesions. A. Colony with fine aerial hyphae. Bar = 540 μm. B. High magnification of fine aerial hyphae. Bar = 140 μm. C. Colony with stout erect thalli. Bar = 585 μm. D. High magnification of stout thalli. Note lateral fine hyphae. Bar = 150 μm.

sporulation using nutrient deprivation (colonies were inoculated into 0.22 μm-filtered seawater). Induction of sporulation was also attempted by incubating the fungus at 15 °C in the dark, and transferring them to near ultraviolet light (black light) for 10–12 hrs/day for 5–7 days each week (F. Dugan, American Tissue-Type Culture Collection, unpublished data).

Statistical Analysis

The Mann Whitney Rank Sum test was used to examine the difference in prevalence of infections between the two species examined. The Spearman Rank Order Correlation test was used to examine the relationship between abalone length, hemolymph parameters, sex and degree of shell mycosis. Differences in the average condition index of infected and uninfected individuals within a species and between the two abalone species used in this study was examined using a students t-test. The Pearson Product Moment Correlation test was used to examine the relationship between the degree of shell mycosis and an abalones' condition index.

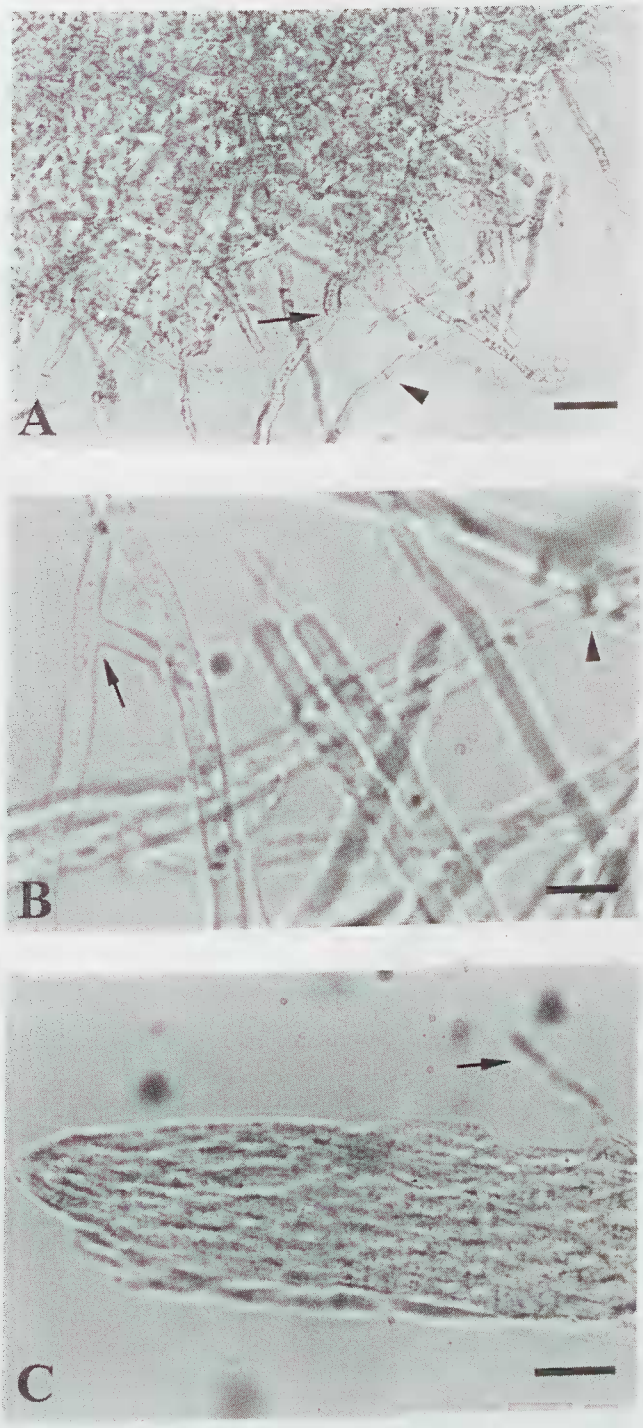

Figure 3 Fungal isolates. Septate (arrow head), branched mycelium (arrow) from a colony with fine aerial hyphae (A) and lateral hyphae from a stout, erect thallus (B). Refractile droplets are denoted with a r Phase contrast. Bars = 10 µm and 4 µm, respectively. C. Thallus from a colony with stout, erect aerial hyphae. A single lateral hypha projects from the main thallus (arrow). Phase contrast. Bar = 16 µm.

Results

Abalone

Abalone lengths ranged from 102.1 to 153.8 mm for *H. iris* and 70.8 to 110.3 mm for *H. australis*. Prevalence of shell mycosis differed between species. However, the difference was not significant ($p>0.05$; N=17). Four of seventeen *H. iris* had moderate (2+) to advanced lesions (3+), and ten of seventeen *H. australis* were infected with early (1+) to advanced (3+) lesions. We observed no significant relationship between shell length and incidence of shell mycosis ($p=0.534$ and $p=0.242$, respectively; N=17 each). The average condition index (CI) of *Haliotis iris* (0.689) differed from that of *H. australis* (0.762) ($p<0.0001$, N=17). However, abalone CI was not influenced by the shell mycosis (*H. iris*: $p=0.240$, $t=-1.23$, DF=14; *H. australis*: $p=0.967$, $t=-0.0417$, DF=14).

We observed a significant relationship between numbers of circulating haemocytes and degree of shell mycosis ($p<0.05$, N=15) in *H. australis* but not in *H. iris* ($p>0.05$, N=14). The number of circulating

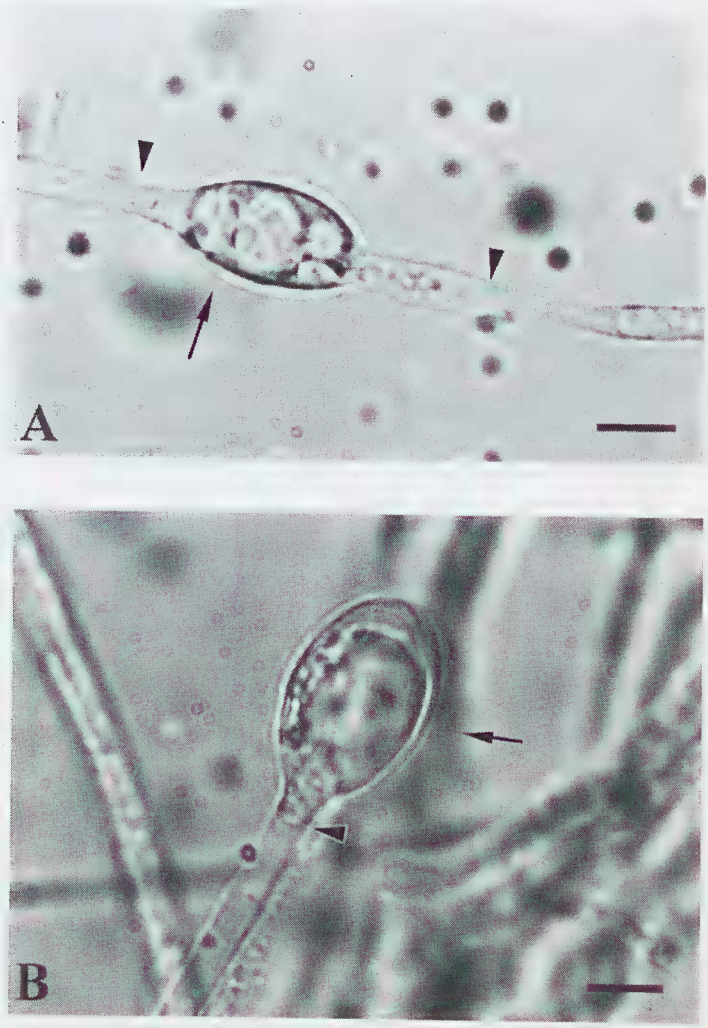

Figure 4 Chlamydospores from a fungal isolate (arrow). Note that the chlamydospore is located between septa (arrow heads, A) or apical to a septum (arrow head, B). Phase contrast. Bars = 4 μm.

haemocytes was elevated in infected *H. australis* (1.19 x 107 cells/ml) relative to uninfected individuals (6.72 x 10^6 cells/ml). No significant relationships were observed between the degree of fungal infection and protein levels of whole hemolymph, serum or sex (immature, male or female) (Spearman Rank Order Correlation: $p<0.050$).

Numerous spherical and polygonal bodies were observed in advanced lesions; many contained fungal hyphae (Figure 1a). We observed numerous sparsely branched, septate fungal hyphae that contained refractile droplets in fresh preparations of both newly established and advanced shell lesions (Figure 1b). Hyphae measured 1.20 – 2.38 µm ($\bar{X}$ = 1.83 µm) in diameter. Occasionally, wider hyphae that measured 2.38 – 3.60 µm ($\bar{X}$ = 2.60 µm) in diameter with an apical swelling that

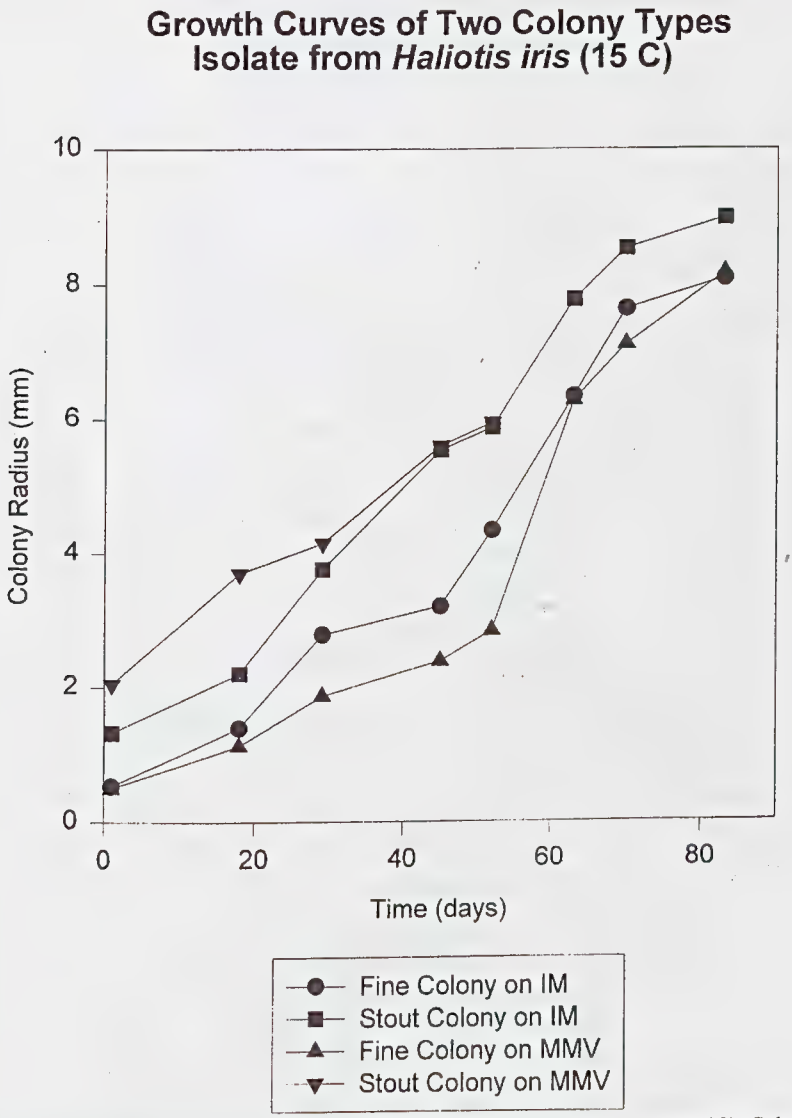

Figure 5 Growth curves of two colony types isolated from an *Haliotis iris* shell lesion (15 °C). Colonies with fine aerial hyphae on IM agar (●) and MMV agar (▲). Colonies with stout, erect aerial hyphae on IM agar (■) and MMV agar (▼).

measured 4.80 – 7.20 x 7.20 – 15.00 µm ($\bar{X}$ = 6.20 x 11.80 µm) were observed within lesions (Figure 1c). Visual examination of the shell, mantle and foot adjacent to lesions showed that the fungi and deposition of conchiolin were limited to the shell (See Grindley *et al.* Submitted for histological examination). Although, *Polydora* sp. infested animals used in our study, no tubes penetrated through to the inner shell surface and none were observed in association with the shell lesions.

Fungal Isolation

Visible growth of aerial hyphae was observed on plates within 12 d at 16 °C on both MMV and IM agars only. Three morphologically indistinguishable isolates were obtained: two originated from

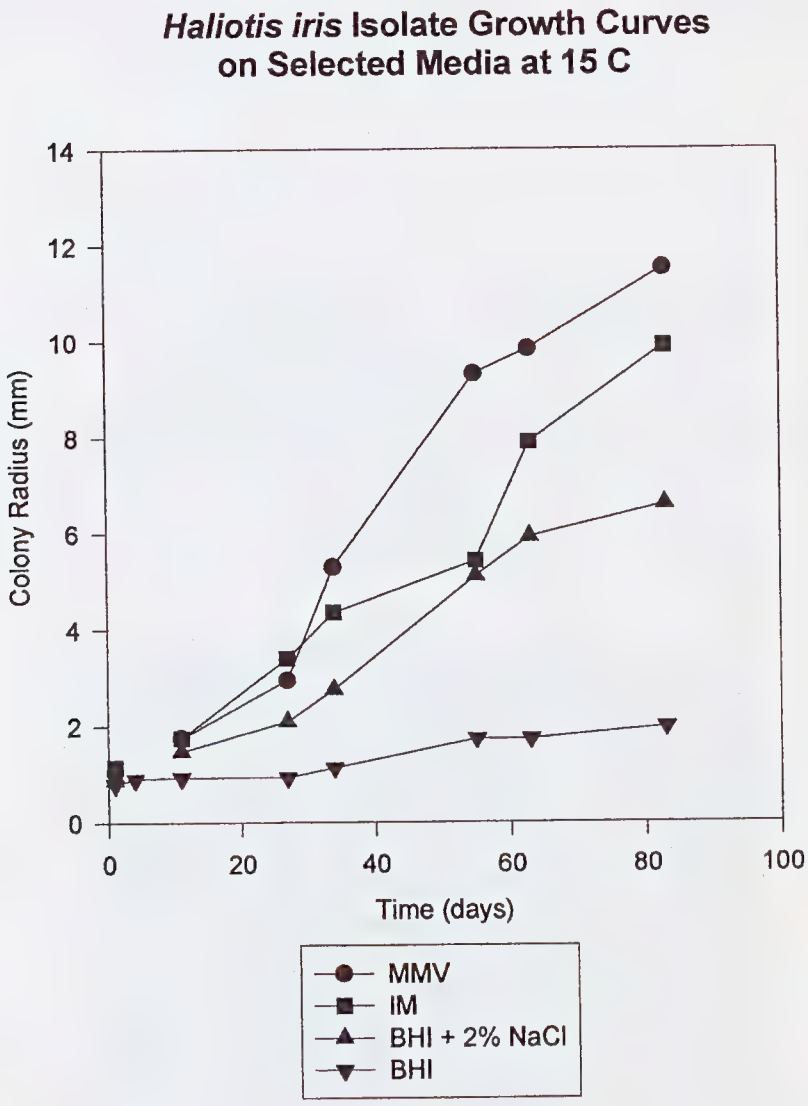

Figure 6 Growth curves of *Haliotis iris* isolate on four media at 15 °C: MMV (●), IM (■), BHI + 2% NaCl (▲), and BHI (▼).

Haliotis iris shells (Hi and DL) and one was cultured from an *H. australis* shell. Unfortunately, the isolate from *H. australis* was lost during passage. Colonies were white to grey in colour with an irregular rhizoid margin. Aerial hyphae were fine to stout and irregular with tapering tips. Fine lateral hyphae projected distally from the primary structure. After passage in the laboratory, colony morphology of both isolates developed into two distinct forms: those with fine aerial hyphae and those with stout, erect, aerial hyphae (Figure 2). Individual colony morphology was retained upon subsequent passage. Fine aerial hyphae were sparsely branched, septate, measured 1.24 – 3.18 µm ($\bar{X}$ = 1.38 µm) in diameter, and contained numerous refractile droplets (Figure 3). Stout aerial thalli, measured 197 – 264 µm at the base and tapered to 2.4 – 7.2 µm at the tip. Lateral mycelia that projected from the stout, erect thalli, measured 1.4 – 4.65 µm ($\bar{X}$ = 2.38 µm) in diameter and were septate, sparsely branched and contained refractile droplets (Figure 3). Chlamydospores formed as

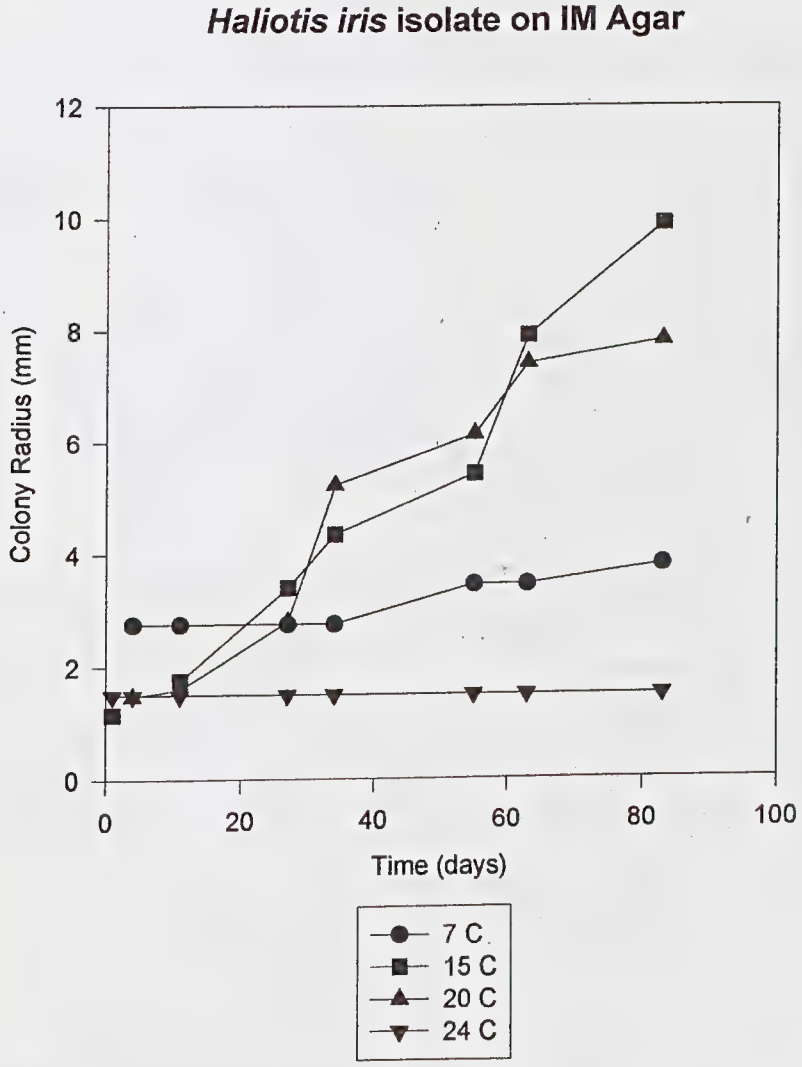

Figure 7 Differential growth of *Haliotis iris* isolate at four temperatures: 7 °C (●), 15 °C (■), 20 °C (▲), and 24 °C (▼).

terminal or intercalary cells, 6.4 μm diameter with refractile bodies, and were observed infrequently upon microscopic examination of both colony types (Figure 4).

Fungal Growth Characteristics

Mycelial growth of both colony types was similar as illustrated in Figure 5. Although both colony types appeared to grow equally well on IM and MMV (Figure 5), repeated examination indicated that the fungus grew best on MMV, followed by IM. Mycelial growth was moderate on BHI+ 2% NaCl and poor on BHI without added salt (Figure 6). Mycelial growth was also poor on CMA and PDA. The fungus grew best at 15 °C, moderate at 19 °C and poor at 7 °C. No growth was observed at 24 °C (Figure 7). All attempts to induce sporulation have failed.

Discussion

We report the first isolation of a fungal pathogen from shell lesions of abalone, *Haliotis* spp. in New Zealand. Shell lesions similar to those described herein have been reported in *H. iris* from New Zealand by Sinclair (1963) and Sainsbury (1977). Sinclair (1963) observed tubercles on the nacreous layer of shells that were composed of alternating layers of conchiolin and nacre and suggested that the lesions were caused by irritation from either sand particles or *Polydora* sp. tubes. Sainsbury (1977) also noted blisters on the nacreous layers of many abalone shells in the Banks Peninsula, New Zealand. Lesions in some animals filled the entire inner surface of the shell and resulted in loss of the shell. Lesions in these animals were thought to be caused by irritation due to boring organisms and/or sediment lodged between the shell and mantle. We did not observe sand particles or *Polydora* tubes in the area of the lesions. In addition, the lesions observed in our study were composed entirely of conchiolin and fungal hyphae and, hence, differed from those reported by Sinclair (1963). Neither Sinclair (1963) nor Sainsbury (1977) reported microscopic examination of shell lesions, particularly early soft lesions as used in our study. Thus, the authors may have overlooked and alternative irritation such as the fungus we consistently observed in all lesions examined.

Fungal lesions in shells of *Haliotis iris*, *H. australis* and *H. virginia* in New Zealand were initially thought to result in decreased value of abalone shells, without an effect on the survival of the animals. However, abalone culturists have experienced losses of farmed animals with lesions while unaffected abalone survived (Grindley 1994). Stress associated with handling and intensive culture conditions may lead to mortality of abalone with shell mycosis as evidenced by observations of Sainsbury (1977) and Grindley *et al.* (Submitted). Although we observed no visual evidence of fungal penetration into live tissues and no differences in condition indices of affected versus unaffected animals, we did observe a significant increase in the number of circulating haemocytes in affected relative to unaffected *H. australis*. The causal relationship between hemocyte numbers and degree of shell mycosis is unclear but may indicate stress or an early response to the disease. The small number of *H. iris* that were infected (N=4) may have attributed to the lack of association between degree of fungal infection and density of circulating haemocytes in this species. An understanding of the pathogenesis of and possible treatments for the shell mycosis is needed.

Fungal-induced shell lesions have been reported in bivalves for several decades (Korringa 1951, Alderman 1985). Ostracoblabe implexa (Class Zygomycotina; Phycomycetes) is the causative agent of shell disease or 'Maladie du pied' of oysters and cockles in Europe and North America. The disease is associated with sea water temperatures above 20 °C and is characterized by the formation of small white spots on the inner surface of the shell that develop into brown warts due to accelerated deposition of conchiolin. Similar to early reports of abalone shell mycosis described herein, definitive proof that the shell disease results in mortality is lacking (Sainsbury 1977; Elston 1994).

Our observation of morphologically similar hyphae in fresh squashes of lesions and the isolation of morphologically indistinguishable colonies from two animals that differentiated into two distinct colony types (stout and fine) suggests that we have isolated a pleomorphic fungus. In addition,

growth characteristics of both colony types was similar as depicted in Figure 5 and fungal morphology is known to change with differing culture conditions (Morrin and Ward 1990; Tucker and Thomas 1994). These data also suggest that the two colony types may be different phenotypes of the same organism. However, all isolates were handled in the same manner in this study and, thus, the possibility exists that the two colony types represent different fungi. Despite this, both colony types were isolated in pure culture from shell lesions of two different abalone in this study which suggests that an association exists between both colony types and abalone shell lesions. *Atkinsiella awabi* (Class Zygomycotina; Phycomycetes) occurs in tissue lesions of stocked abalone, *Haliotis sieboldii*, in Japan. The fungus we isolated from shell lesions differed in nearly all characteristics from *A.* awabi as described by Kitanchareon *et al.* (1994). *Atkinsiella awabi* is a nonseptate fungus, with an irregular thallus, and is larger in diameter than the fungus reported herein (16–41 µm versus 1.24–4.65 µm, respectively). In addition, *A. awabi* readily sporulates, grows best at 20 °C and grows equally well on CMA and a medium similar to MMV. In contrast, the fungus we isolated is septate with a regular thallus, does not readily sporulate, grows best at 15 °C and growth on MMV far exceeds that on CMA. However, both organisms are branched and require salt for growth. The taxonomic placement of the fungus reported herein is uncertain. Due to lack of sexual structures, the fungus reported herein is provisionally placed in the Class Deuteromycotina (Fungi Imperfecti, Jawetz *et al.* 1987). Morphological characteristics of the vegetative mycelium cultured from shell lesions resembled those of hyphae within lesions (Figures 2 & 4) and suggests that the fungus described in our study is the causative agent of the shell lesions. We are currently attempting to reproduce shell lesions in abalone in our laboratory.

Acknowledgments

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